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14. ABSTRACT

In this study we investigated the relationship between Pten, Tsc2 and the NMDA receptor using conditional knock out mice. We originally hypothesized that loss of Tsc2 could alter NMDA receptor expression through a mechanism that may involve Pten downregulation, rather than changes in mTOR signaling. The data generated during the course of this study conclusively demonstrate that *Pten* loss in the forebrain leads to alterations in the expression of all major NMDA receptor subunits. However, Tsc2 loss in the same brain cells does not affect the NMDA receptor. Also, we found that Tsc2 loss does not affect Pten levels. Taken together, our findings suggest that Pten controls NMDA receptor expression through a mechanism that is independent of mTOR signaling and is not affected by Tsc2 activity. Further studies will be required to determine whether Pten affects NMDA receptor expression through direct protein-protein interaction, or through Akt-dependent signaling. Overall, our results suggest that NMDA receptor abnormalities do not play a central role in cognitive defects in TSC patients.

15. SUBJECT TERMS
Pten, NMDAR, TSC

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INTRODUCTION

Tuberous Sclerosis Complex (TSC) is a genetic disease resulting from the loss of either the *TSC1* or the *TSC2* genes. These genes produce proteins that inhibit the growth-promoting kinase complex mTORC1. This mechanism may account for tumor susceptibility, however, it is not clear whether it underlies cognitive dysfunction in TSC patients. Loss of Pten, another tumor-suppressor gene that functions upstream of TSC, also causes mTORC1 activation. We previously discovered that *Pten* loss affects the expression of NMDA receptor subunits, a neurotransmitter receptor that is very important for learning and cognition. Since studies of human tissue reported alterations in NMDA receptor subunits in TCS patients (Talos et al., 2008), we wondered whether disrupted synaptic function and cognition in these patients may be due to altered NMDA receptor expression. Thus, we planned to generate brain-specific conditional *Tsc2* mouse mutants, and to examine and compare NMDA receptor subunit expression in *Pten* and *Tsc2* mutants. We also set out to investigate the molecular mechanisms of Pten- and Tsc2-dependent NMDA receptor regulation. In addition to the shared activation of mTORC1, a published study indicated that *Tsc2* loss may upregulate Pten expression (Mahimainathan et al., 2009). Thus, we worked to determine whether the deregulation of NMDA receptor expression in *Pten* or *Tsc2* mutants is dependent on increased mTORC1 activity or to a different Pten-dependent biochemical activity. In this study, we were successful in generating novel brain-specific *Pten* and *Tsc2* conditional knock out mouse lines, and we were able to examine the expression of NMDA subunits in the brain of these mutants. However, we found that only the loss of *Pten* affects the expression of NMDA receptor subunits, whereas the loss of *Tsc2* does not. Since the loss of both genes results in the activation of mTORC1 signaling, we concluded that NMDA receptor subunit expression is not dependent on this kinase complex, but is mediated by a different molecular mechanism that is uniquely affected by Pten.

BODY

During this entire award period we completed the work proposed under *Specific Aim 1: to examine the subunit composition of the NMDA receptor in Pten and Tsc2 knockout mice*, and also addressed the *Specific Aim 2: to determine whether the receptor abnormalities in Tsc2 mutants are dependent on the elevated activity of PTEN or mTOR*. In the first year we generated novel NS-Tsc2 conditional knock out mice, and compared the expression of NMDA receptor subunits with NS-Pten mutant mice. However, the extent of the gene deletion in these lines was insufficient to detect activation of the signaling pathway. Thus, in the second year of support we revised our genetic strategy, generated NEX-Tsc2 knock out mice, and compared the expression of NMDA receptor subunits with NEX-Pten mutants. The goal of the work, to compare the effects of *Pten* and *Tsc2* gene deletions on NMDA receptor expression, did not change. The results of our experiments are described in detail below according to the proposed tasks.

Task 1. Establish the NMDA receptor profile of NS-*Pten* mutant mice (completed in year 1):

1a. Breeding of Cre+, Pten heterozygous mice to generate wild type, heterozygous and homozygous mutant mice.

We used conditional knock out mice in which deletion of the Pten gene occurs in a neuronal subset (NS) due to the expression of a 'driver' GFAP-Cre transgene (Kwon et al., 2001). We intercrossed NS-Cre+/+;Pten wt/fl mice and generated homozygous (Cre+/+;Pten fl/fl), heterozygous (Cre+/+;Pten wt/fl) and wild type (Cre+/+;Pten wt/wt) mice at postnatal day (P) 21. We chose this age to avoid the confounding effect of spontaneous seizures, which typically begin around 1 month of age as shown in our previous studies (Ljungberg et al., 2009).

1b. Dissection of the hippocampus, protein extraction and Western blot analysis of NMDA receptor subunits.

We euthanized 3 sets of homozygous (KO), heterozygous (Het) and wild type (Wt) NS-Pten mice, dissected the hippocampus and prepared whole tissue protein homogenate (Hom) as well as crude synaptosomal fractions (P2) as previously described (Ventruti et al., 2011). We then proceeded to analyze the levels of NMDAR subunits NR2A, NR2B and NR1 in homogenate and synaptosomal fractions by Western blotting (Fig. 1). All subunit levels were normalized to actin, and the data were statistically analyzed using the one-way ANOVA test. Contrary to our expectation, the results indicate that there are no significant differences between genotypes.

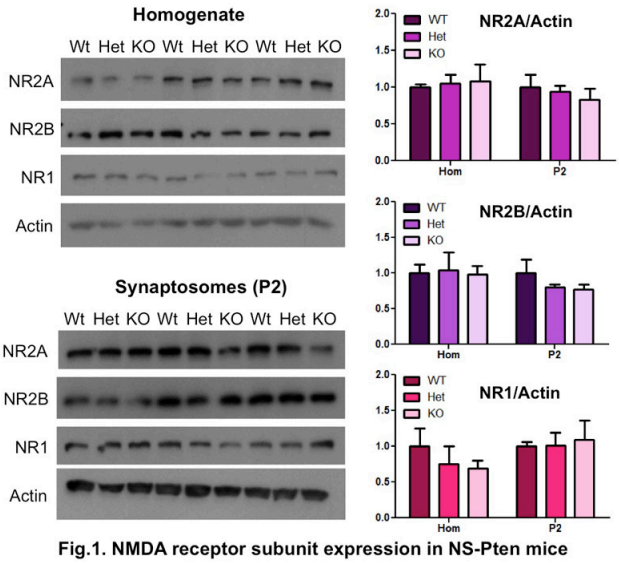

Fig.1. NMDA receptor subunit expression in NS-Pten mice

To ensure that Pten deletion and activation of the PI3K/mTOR signaling pathway had occurred at least in homozygous mutants, we performed Western blot analysis of Pten, PI3K and mTOR targets such as phosphoAkt (for mTORC2) and phosphoS6 (for mTORC1) (Fig. 2). The blots were reprobed for total Akt, total S6 and actin to normalize for protein content in each lane. Unlike previous studies utilizing adult mice (Kwon et al., 2003), the present results indicate that the levels of Pten at P21 were only modestly reduced in homozygous mutants. The levels of phosphoThr308-Akt (pAkt(T)) were significantly increased in KO mice, indicating a significant increase in PI3K signaling. However, the levels of phosphoSer473-Akt (pAkt(S)), a target of mTORC2, and phosphoSer240/244-S6 (pS6), a downstream target of mTORC1 were only marginally or not affected at all. These data suggest a partial expression of NS-Cre in the hippocampus of NS-Pten mice at P21.

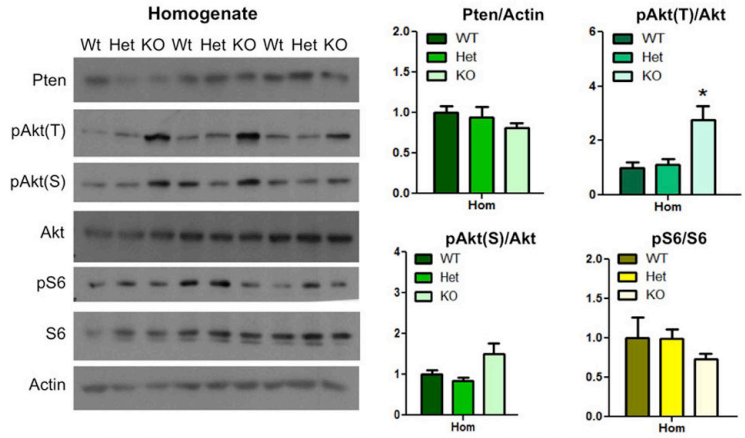

Fig. 2. Pten expression and PI3K/mTOR signaling in NS-Pten mice

Task 2. Regulatory review and approval of animal protocol (completed in year 1):

We generated a novel NS-*Tsc2* conditional knock our mouse line. These mice were added to our protocol with the approval of IACUC.

Task 3. Establish the NMDA receptor profile of NS-*Tsc2* mice (completed in year 1):

3a. Breeding of homozygous floxed mice with hGFAP-Cre positive mice to generate an NS-Tsc2 colony.

We obtained floxed *Tsc2* mice described in (Way et al., 2009), and crossed them to NS-Cre+/+ mice. The initial matings of *Tsc2* fl/fl males with NS-Cre +/+ females generated F1 progenies consisting of double heterozygous NS-Cre+/-;*Tsc2* wt/fl mice. Some of these mice were backcrossed to NS-Cre +/+ mice to generate the preferred breeders (NS-Cre+/+;*Tsc2* wt/fl) for

colony maintenance.

3b. Breeding of Cre+, *Tsc2* heterozygous mice to generate wild type, heterozygous and homozygous mutant mice.

While waiting for the generation of optimal NS-Cre+/+;*Tsc2* wt/fl, we proceeded to backcross F1 double heterozygous NS-Cre+/-;*Tsc2* wt/fl mice with the original Tsc2 fl/fl males. This mating scheme generated, as expected, approximately 25% homozygous mutants (NS-Cre+/-;*Tsc2* fl/fl), 25% heterozygous mutants (NS-Cre+/-;*Tsc2* wt/fl), and 50% wild type controls (NS-Cre-/-;*Tsc2* fl/fl or NS-Cre-/-;*Tsc2* wt/fl) (Table 1).

Mouse Type	Genotype	n of mice born	% of total	% expected
Homozygous mutant	Cre -/-;Tsc2 fl/fl	16	24	25
Heterozygous mutant	Cre +/-;Tsc2 fl/wt	17	25	25
Control	Cre -/-;Tcs2 fl/fl	22	33	25
	Cre -/-;Tsc2 fl/wt	18	18	25
	Total	40	51	50

Table 1. NS-Tsc2 mice born in 9 litters (67 mice total) from F1 backcross matings

However, many of the homozygous mutants died prematurely between postnatal day 8 and 15. When a cohort of 16 homozygous mutants was observed, only 1/3 survived until P19 (Fig. 3). No premature death was observed in heterozygous mutants or controls.

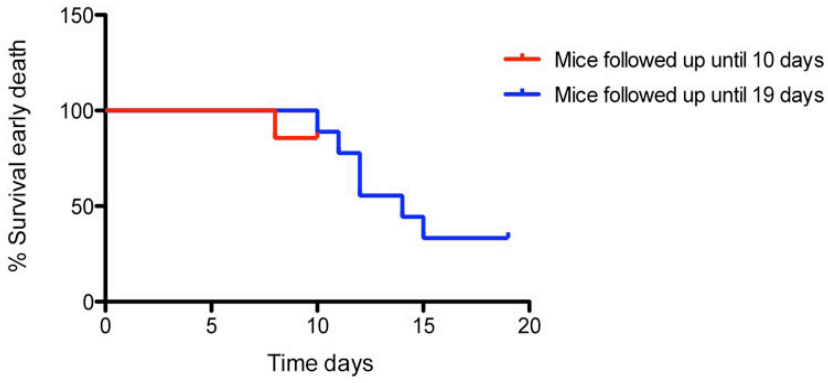

Fig. 3. Early lethality of homozygous NS-Tsc2 mice

3c. Dissection of the hippocampus, protein extraction and Western blot analysis of NMDA receptor subunits.

Because of the unexpected early lethality of homozygous NS-*Tsc2* mice, we sampled a cohort of NS-*Tsc2* mice at P10. First, we dissected the neocortex (CX), hippocampus (HC), and cerebellum (CB) of 2 NS-*Tsc2* homozygous (KO) and 1 control mice (C) and analyzed NMDAR NR2 subunits (Fig. 4). The data show that there are no differences between mutants and control.

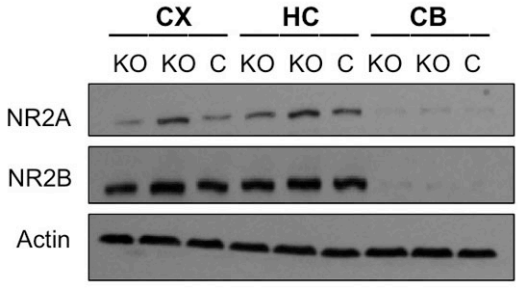

Fig. 4. NMDAR subunits in NS-Tsc2 mice

Next, we checked for *Tsc2* loss and PI3K/mTOR activation by Western blotting in a larger set of P10 littermates. The data show that there is no detectable loss of *Tsc2* or activation of the signaling pathway in homozygous or heterozygous mutant mice in any of the analyzed brain structures at this age (Fig. 5).

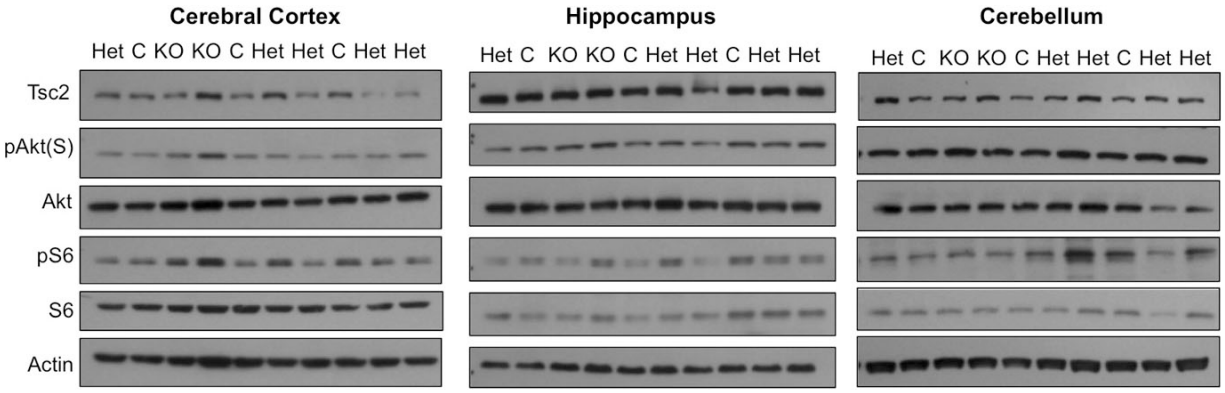

Fig. 5. Tsc2 and PI3K/mTOR signaling in a litter of P10 NS-Tsc2 mice

Thus, our Western blot analysis failed to identify defects in NMDA receptor subunit expression in NS-*Tsc2* mice as well as in NS-Pten mice. However, because the data also failed to detect the expected gene deletion and mTOR signaling activation in mutant mice (particularly in younger NS-*Tsc2* mice), we hypothesized that the expression of Cre in these NS lines may be too low at the ages analyzed to cause detectable effects. Therefore, we conducted an additional experiment where we crossed the original NS-Cre driver line with a tdTomato fluorescent reporter line (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J) we obtained from the Jackson Laboratories. The F1 progeny (NS-Cre+/-; tdTomato+/-) was sacrificed at P14 and P30, and brain sections were imaged using a fluorescence microscope (Fig. 6). The image (overlaid with the DAPI nuclear stain, blue) shows that the tdTomato reporter (red) is expressed in few cells of the cerebral cortex and cerebellum, and is limited to the dentate gyrus in the hippocampus at P14. At later time points (P30), the reporter is expressed in many more cells of the cerebral cortex and cerebellum, although it remained limited to the dentate gyrus in the hippocampus.

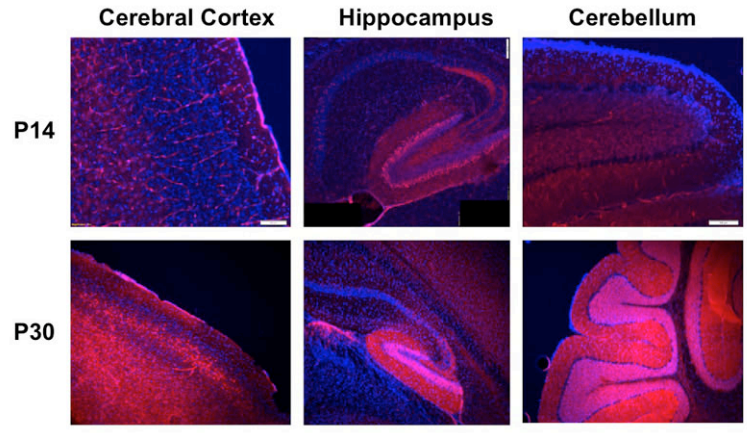

Fig. 6. tdTomato reporter expression NS-Cre mice

To further investigated mTOR signaling at a cellular level, we sectioned the brain of an additional set of homozygous NS-*Tsc2* mutant and a control littermate at P10, and conducted pS6 immunofluorescence. The data reveals that overall mTOR activation is not significantly increased in mutant mice at this age, except perhaps for few isolated cells.

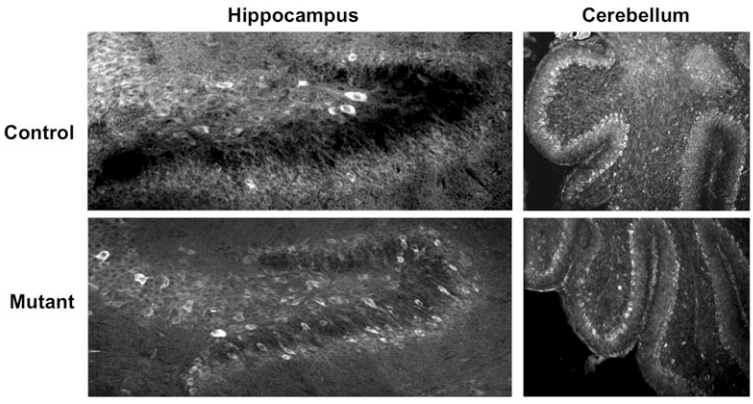

Fig. 7. pS6 expression P10 NS-Cre mice

These findings indicated that NS-Cre driven conditional

mutants are not suitable for the proposed studies, and that cell population analysis, such as Western blotting, are not informative in developing mice. Adult time points could not be collected in NS-*Tsc2* mice due to early lethality of the mutants, and in NS-*Pten* due to spontaneous seizures. Therefore, we modified our original research strategy for the second year of support, and conducted the revised Tasks described below.

New Task 4: To complete the analysis of mTOR signaling and NMDA receptor subunits in heterozygous and homozygous NEX-*Pten* mice (completed in year 2):

We previously used the NEX promoter-driven Cre transgenic line (Goebbels et al., 2005) to generated a NEX-*Pten* conditional knock out mouse line. This genetic strategy results in the deletion of the *Pten* gene in virtually all the newly-generated excitatory neurons of the forebrain (Kazdoba et al., 2012). We found that homozygous mutants express elevated NR2A and NR2B subunits in the perinatal forebrain (Kazdoba et al., 2012). During the second year of support, we further analyzed that the obligatory NMDA receptor subunit NR1 and found that it is also expressed at higher levels in homozygous NEX-*Pten* mutants (Fig. 8). We also analyzed heterozygous mutant samples and found that they express intermediate levels of all subunits (Fig. 8).

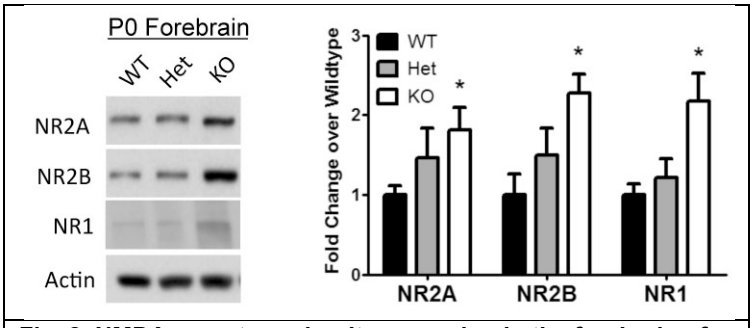

Fig. 8. NMDA receptor subunit expression in the forebrain of newborn NEX-Pten mice

However, only homozygous mutant were significantly different from wild type levels (ANOVA, n=3 mice per genotype, p<0.05).

To determine whether NMDA receptor changes were unique to this class of glutamate receptors we also analyzed AMPA receptors GluR1 and GluR2/3. We found that GluR2/3, but not GluR1 levels, were significantly increased in homozygous NEX-Pten samples (Fig. 9) (ANOVA, n=3 mice per genotype, p<0.05). Finally, to confirm gene deletion and activation of the PI3K/mTOR pathway we also probed similar Western blots with Pten, pAkt(T), pAkt(S) and pS6 antibodies, and measured the ratio of phosphorylated to total proteins. We found that, as expected, Pten levels were significantly reduced in homozygous samples, resulting in an increase in Akt and S6 phosphorylation, which indicates activation of the signaling pathway in NEX-*Pten* mutants (Fig. 10) (ANOVA, n=3 mice per genotype, p<0.05).

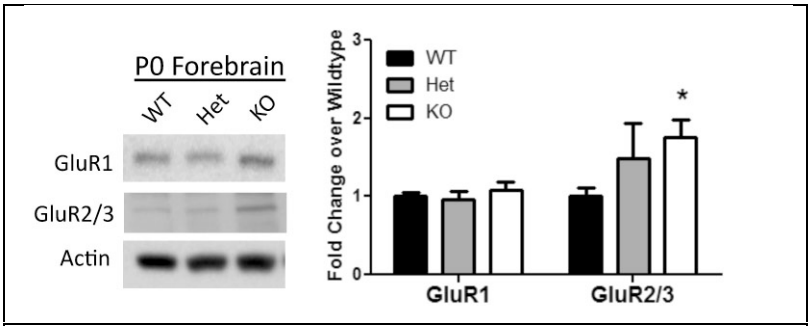

Fig. 9. GluR subunit expression in the forebrain of newborn NEX-Pten mice

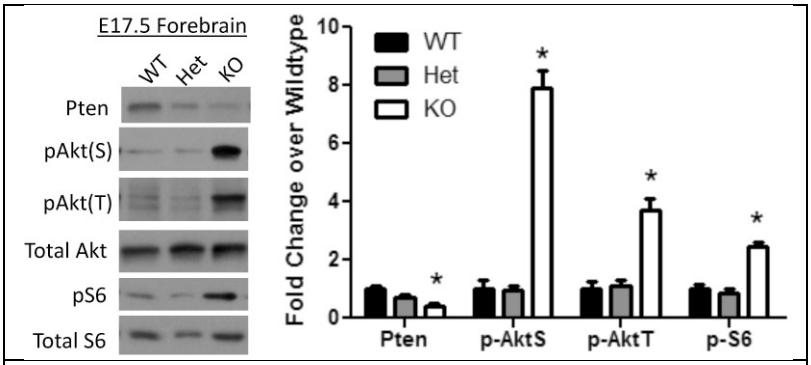

Fig. 10. Signaling abnormalities in the forebrain of NEX-Pten mutant mice

New Task 5: To analyze mTOR signaling and NMDA receptor subunits in heterozygous and homozygous NEX-Tsc2 mice (completed in year 2):

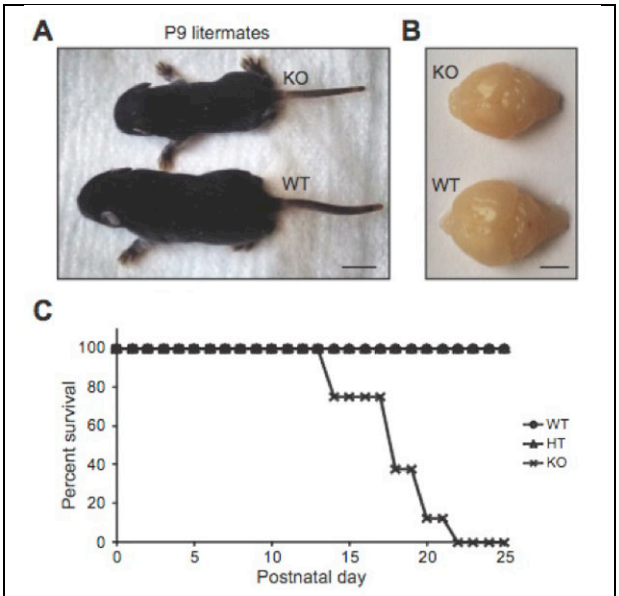

Fig. 11. Nex-*Tsc2* mice. *A*, Animal appearance. *B*, Brain appearance. *C*, Survival plot.

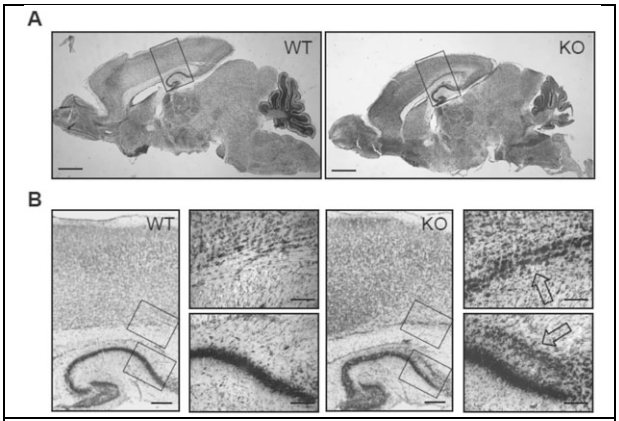

Fig. 12. Neuroanatomy of Nex-*Tsc2* mice at P10. *A*, Whole brain. *B*, Magnified regions of the neocortex and hippocampus.

Using the same Cre driver line used to generate NEX-Pten mice, and floxed *Tsc2* mice, we first generated a new NEX-*Tsc2* conditional mutant line. The colony was established by interbreeding double-heterozygous $Cre^{+};Tsc2^{flox/wt}$ mice. This breeding strategy also generates wild type controls ($Cre^{+};Tsc2^{wt/wt}$) and heterozygous mutants ($Cre^{+};Tsc2^{flox/wt}$). NEX-*Tsc2* heterozygous mice (HET) appeared indistinguishable form wild type controls (WT), and exhibited a normal life span. Homozygous mice (KO) were born according to a Mendelian inheritance ratio and appeared indistinguishable from control littermates until around postnatal day (P) 5. Thereafter, however, they failed to thrive at the same rate as littermate controls and appeared runt (Fig. 11). By P10 the body weight of KO mice was significantly reduced (69.6 ± 6.3% of WT control, $p<0.001$), and most KO mice died during the third postnatal week (Fig. 11). Although no overt seizure activity was observed in KO mice, it seems likely that death is caused by massive synaptic dysfunction. We also performed a preliminary histological analysis of the brain in NEX-*Tsc2* mice. Brain sections were obtained from P10 WT, HET and KO littermates, and stained with thionin. The brain of HET mice appeared indistinguishable from that of WT mice. The brain of KO mice appeared slightly smaller, but no gross cortical malformations were noted (Fig. 12). However, higher magnification of light microscopy images revealed subtle alterations in the cortical and hippocampal cytoarchitecture. The presence of ectopic cells was noted in the deep regions of the cerebral cortex and in the stratum oriens of hippocampus (Fig. 12, arrows). Furthermore, some thickening and dispersion of the hippocampal pyramidal cell layer and of the dentate gyrus was observed.

We then performed a Western blot analysis of forebrain lysates from WT, HET and KO NEX-*Tsc2* mice at P10. We examined the levels of Tsc2, and the phosphorylation levels of components of the Akt/mTOR signaling pathway, including phospho-Akt (Ser473 and Thr308) and phosphoS6. All proteins examined appeared normal in HET mice, despite the reduction in gene dosage (Fig. 13). However, the levels of Tsc2 were significantly reduced in KO mice (ANOVA, $n=3$ mice per genotype, $p<0.05$). This defects correlated with increased levels of phosphoS6, likely reflecting activation of mTORC1, and decreased levels of phospho-Akt, reflecting reduced PI3K and/or mTORC2 activity via a negative feedback mechanism. These findings are consistent with the loss of TSC activity. Interestingly, we also noted that

levels of Pten were not decreased in the forebrain of NEX-*Tsc2* mutant mice (Fig. 13), contrary to a published report using embryonic fibroblasts (Mahimainathan et al., 2009), suggesting that negative feedback regulation does not occur in this organ.

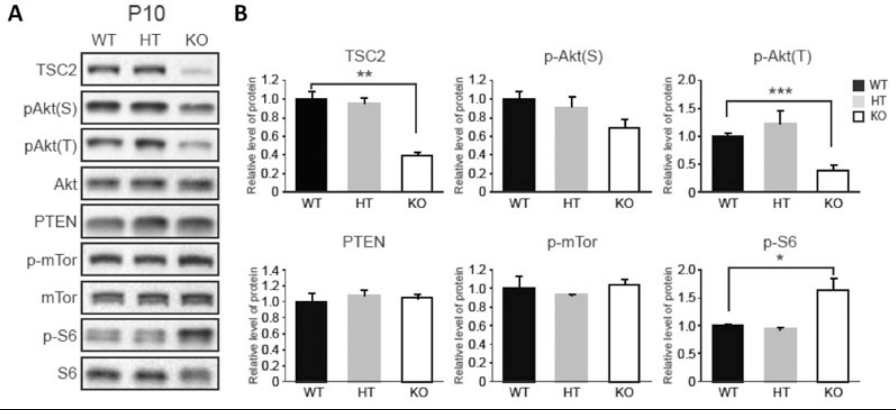

Fig. 13. Western blot analysis of Tsc2 levels and Akt/mTOR signaling in Nex-*Tsc2* mice at P10. *A*, Representative blots. *B*, Quantification from *n*=3 mice/genotype.

Finally, we analyzed the expression of NMDA and AMPA receptor subunits in the forebrain of wild type, heterozygous and homozygous NEX-*Tsc2* mice at P10. Our data indicate that none of the NMDA receptor or GluR subunits analyzed was significantly affected in either, heterozygous or homozygous NEX-*Tsc2* mice (Fig. 14) (ANOVA, *n*=3 mice per genotype, *p*>0.05).

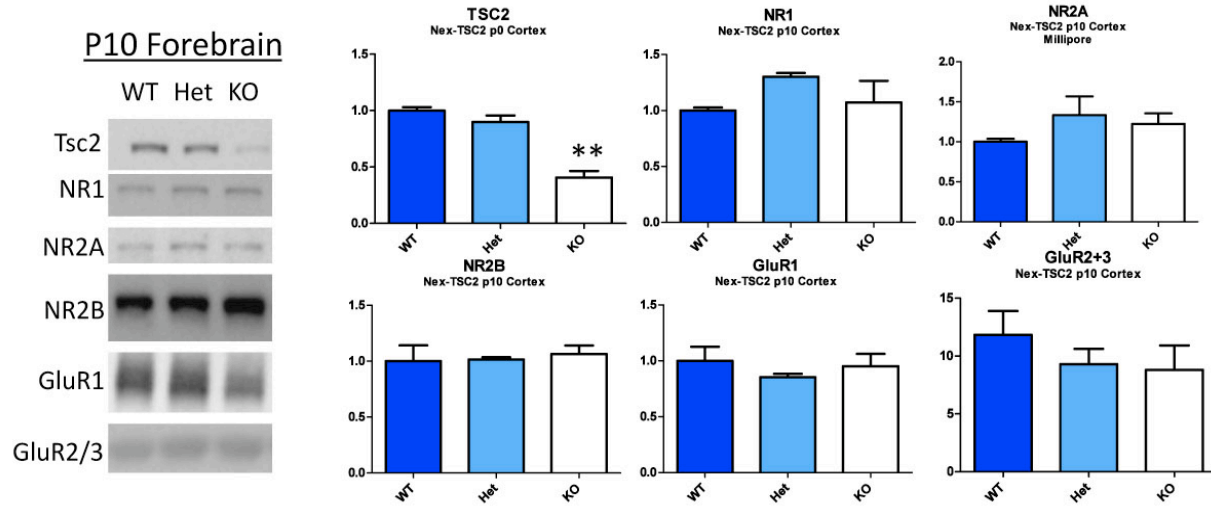

Fig. 14. Western blot analysis of NMDA receptor and GluR levels in Nex-*Tsc2* mice at P10.

Together, our data demonstrate that *Tsc2* loss does not affect the expression of these receptors, suggesting that mTORC1 or mTORC2 do not mediate these events in response to *Pten* gene deletion in NEX-*Pten* mutant mice.

<u>New Task 6. To examine mTORC1 activity and NMDA receptor expression in NEX-Tsc2 and NEX-Pten mutant mice treated with rapamycin (not performed).</u>

Given the results of Task 5 above, we did not proceed to treat mutant mice with the mTORC1 inhibitor rapamycin. Also, since Pten levels are unaffected, and Akt activity is downregulated in NEX-*Tsc2* mutants, we did not attempt to treat these mice with Pten or Akt inhibitors as we had originally envisioned.

KEY RESEARCH ACCOMPLISHMENTS

In this exploratory study we accomplished the following:

1. We analyzed NS-*Pten* mice for gene loss, PI3K/mTOR signaling and NMDA receptor expression at a late postnatal (P21) age by Western blotting. However, we found partial *Pten* loss and partial activation of the PI3K/mTOR signaling, and no abnormalities in the NMDA receptor, likely due to insufficient gene ablation by the NS-Cre driver transgene. This line was thus discontinued.
2. We generated a new NS-*Tsc2* line. Mutant mice died prematurely during the second postnatal week. Similarly to NS-*Pten* mice, we found partial *Tsc2* loss and activation of the mTOR signaling, and no abnormalities in the NMDA receptor, possibly due to insufficient gene ablation by the NS-Cre driver transgene. This line was discontinued.
3. We then switched to the more effective NEX-Cre driver transgene, and analyzed NEX-*Pten* mutant mice. We found significant gene loss, increased PI3K/mTOR signaling and increased expression of all NMDA receptor subunits analyzed, plus increased levels of the AMAP receptor subunit GluR2/3.
4. We therefore generated and analyzed novel NEX-*Tsc2* mutant mice. We found significant gene loss, decreased Akt activity, increased mTOR signaling, but no changes in the expression of all NMDA or GluR receptor subunits analyzed.

REPORTABLE OUTCOME

Manuscripts

- Lee GH, Crowell BC, Kazdoba TM and D'Arcangelo G. Complex neurological phenotype in mutant mice lacking *Tsc2* in excitatory neurons of the developing forebrain (in preparation).

Abstracts

- Kazdoba TM, Sunnen CN, Crowell B, Lee GH, Anderson AE, D'Arcangelo G. Development and characterization of NEX-Pten, a novel forebrain excitatory neuron-specific knockout mouse. Program #/Poster # 548.23/G44. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.
- Dal Pozzo V, Crowell B, Lee GH, D'Arcangelo G. Complex neurological phenotype in mutant mice lacking Tsc2 in excitatory neurons of the developing forebrain. Program#/Poster# 519.16/Y17. 2014 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2014. Online.

Presentations at scientific conferences or seminar series

- Kazdoba TM, Sunnen CN, Crowell B, Lee GH, Anderson AE, and D'Arcangelo G. Examination of the Role of Pten in Ionotropic Glutamate Receptor Expression. National Graduate Student Research Conference, October 2012, Bethesda, MD. (poster)
- D'Arcangelo G. PI3K and mTOR signaling in brain injury and disease. Shriner's Hospital Pediatric Research Center/Temple University School of Medicine seminar series, September 2014. (lecture)

Degrees obtained during this award

- Kazdoba, Tatiana M., PhD awarded in December 2013. Thesis title: Role of Reelin and PTEN in synapse formation and NMDA receptor expression. Dr. Kazdoba is currently a

postdoctoral trainee at the University of California Davis where she received a postdoctoral fellowship from the MIND Institute.

Animal models generated during this award

- NEX-*Tsc2* conditional knock out mouse line.

Grant applications based on this award

- Idea Development Award GRANT 11709141 (D'Arcangelo, PI)
  DOD-CDMRP-TSCRP
  Cell type-specific contributions to the TSC neuropathology
  Project period: 07/01/15- 06/30/18
  Annual direct costs: $155,574
  Annual total costs: $232,442
  The goal of this work is understand the cellular and molecular mechanisms that cause neurological defects in TSC patients. In particular we will characterize the neuroglial phenotype of a conditional mouse model lacking Tsc2 specifically in excitatory neurons of the forebrain (NEX-Tsc2).

List of personnel receiving support from this award

- Gabriella D'Arcangelo (PI)
- Tatiana M Kazdoba (graduate student)

CONCLUSIONS

The goal of this proposal was to investigate the relationship between Pten, Tsc2 and the NMDA receptor using conditional knock out mice. Based on the data available in 2011 we had hypothesized that loss of Tsc2 could alter NMDA receptor expression through a mechanism that may involve Pten downregulation, rather than changes in mTOR signaling (Fig. 15A). The data generated during the course of this study conclusively demonstrate that *Pten* loss in the forebrain leads to alterations in the expression of all major NMDA receptor subunits. However, Tsc2 loss in the same brain cells does not affect the NMDA receptor. Also, we found that Tsc2 loss does not affect Pten levels. Taken together, our findings suggest that Pten controls NMDA receptor expression through a mechanism that is independent of mTOR

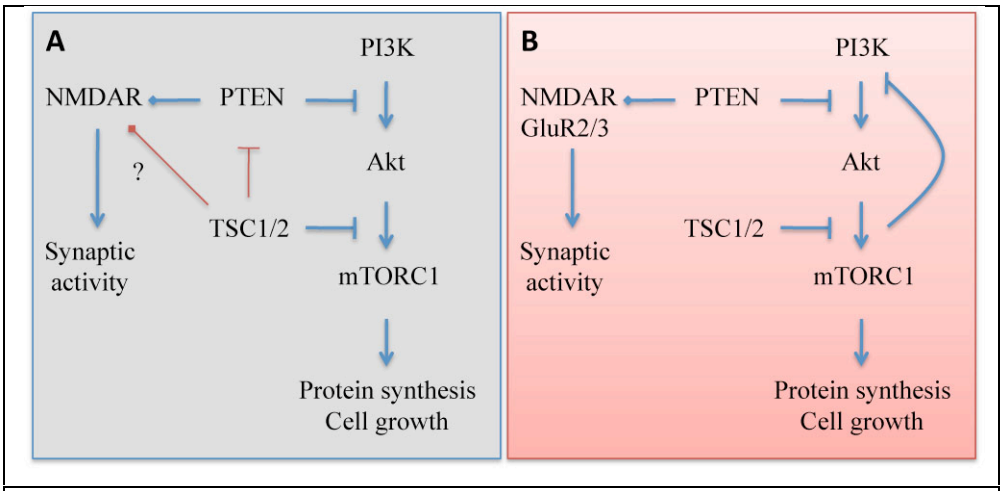

Fig. 15. A, original model of the relationship between Pten, Tsc2 and the NMDA receptor. B, revised model.

signaling and is not affected by Tsc2 activity (Fig. 15B). Further studies will be required to determine whether Pten affects NMDA receptor expression through direct protein-protein interaction, or through Akt-dependent signaling. Overall, our results suggest that NMDA receptor abnormalities do not play a central role in cognitive defects in TSC patients.

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